Granulocyte-macrophage colony-stimulating factor-dependent CD11c-positive cells differentiate into active osteoclasts

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Abstract

Objective. Levels of circulating cytokines are elevated in inflammatory diseases. Previously, it was shown that interleukin (IL-)17A, in synergism with 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and tumor necrosis factor α (TNFα), induces the release of granulocyte-macrophage colony-stimulating factor (GM-CSF) by murine osteoblasts *in vitro*. In this study, we further analyzed the effects of GM-CSF on osteoclast development *in vitro*.

Methods. The effects of IL-17A, TNFα, and 1,25$(OH)_2D_3$ on the regulation of osteoclast development were investigated in cocultures of bone marrow-derived osteoclast progenitor cells (OPC) and mouse calvarial osteoblasts. Additionally, OPC were grown for 3 days in media containing macrophage colony-stimulating factor (M-CSF), GM-CSF, or M-CSF/GM-CSF. Subsequently, the osteoclastogenic potential and the capacity to dissolve amorphous CaP were assessed in each of the three populations of OPC.

Results. IL-17A, in synergism with TNFα and 1,25$(OH)_2D_3$, inhibited the development of osteoclasts in the cocultures by stimulating the osteoblasts to release GM-CSF. GM-CSF-treated OPC expressed traits characteristic of dendritic cells. Upon removal of GM-CSF and supplementation of the culture media with M-CSF/ and RANKL, the cells lost their dendritic cell characteristics and differentiated into osteoclasts. OPC pretreated with GM-CSF and M-CSF/GM-CSF exhibited delayed development to osteoclasts and an extended proliferation phase.

Conclusion. Elevated levels of GM-CSF in systemic inflammatory diseases may cause an expansion of the OPC pools in the bone, bone marrow, and blood. Upon homing to bone, this may lead to an increase in the number of osteoclasts and in bone resorption.

Introduction

Rheumatoid arthritis (RA) is an inflammatory disease of the human skeleton. The imbalance between pro-and anti-inflammatory cytokines in affected joints leads to infiltration of the synovium by immune cells, cartilage damage, and loss of subchondral bone. These bone erosions are governed by pro-inflammatory cytokines, which shift the balance between osteoblastic and osteoclastic activities towards resorption (1, 2). Osteoclasts are multinucleated cells of haematopoietic origin and form by fusion of monocyte/macrophage lineage progenitor cells. Two growth factors, haematopoietic growth factor macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL), are essential and sufficient for osteoclastogenesis. RANKL binds to RANK on the surface of haematopoietic progenitor cells, activating a signal cascade through tumor necrosis factor receptor-associated factors (TRAFs) that leads to the downstream activation of inhibitor of NF-κB kinase (IKK), c-Jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK), and Src (2). Binding of RANKL to RANK is blocked by osteoprotegerin (OPG), a decoy receptor for RANKL. Physiologically, the RANKL:OPG ratio governs the biological efficacy of RANKL and its action on osteoclastogenesis. Besides M-CSF and RANKL, numerous cytokines, such as tumor necrosis factor alpha (TNFα), contribute to the modulation of osteoclast development. TNFα stimulates osteoclast differentiation indirectly by modulating the RANKL and OPG levels in stromal cells and directly by acting on osteoclast progenitor cells (OPC) in synergism with RANKL (3, 4). *In vitro*, osteoclasts differentiate from bone marrow cells (BMC) grown in culture media supplemented with M-CSF and RANKL, or in coculture of BMC or OPC and osteoblast lineage cells. Upon stimulation with 1,25-

dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], RANKL is upregulated in mesenchymal cells, while OPG levels are attenuated (5, 6).

In the microenvironment of the bone marrow, immune cells interact with bone cells to modulate their differentiation and activity. In the inflamed joints of patients with RA, activated T-cells secrete osteoclastogenic cytokines, including soluble RANKL, TNFα, interleukin (IL-)6, granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-17A (1, 7, 8). Furthermore, T-cells trigger macrophages, fibroblasts, and endothelial cells to release pro-inflammatory cytokines, resulting in increased levels in the synovial fluid (9-11). TNFα, IL-1, and IL-17A are targeted in RA therapies (11); inhibitory antibodies against these cytokines and combinations thereof attenuate both synovial inflammation and subchondral bone resorption (12, 13).

TNFα and IL-17A are potent inducers of GM-CSF release by mesenchymal cells (14, 15). GM-CSF is a member of the family of haematopoietic growth factors, which were originally identified by their capacity to induce colony formation in granulocytes and macrophages *in vitro* (16, 17). M-CSF and GM-CSF control the proliferation, differentiation, activation, and survival of monocyte/macrophage lineage cells (17). Studies on M-CSF-deficient *op/op* mice demonstrated this factor to control the development of most non-inflammatory macrophage populations and osteoclasts. M-CSF-independent macrophages, mainly involved in inflammatory and immune responses, are not affected by the *op/op* mutation (18, 19).

M-CSF is constitutively expressed by multiple cell types and circulates in the blood. GM-CSF is not detectable in physiological conditions, but levels rise dramatically during immune response and inflammation (17). Besides its growth factor-like action in

granulocytic cells and macrophages, GM-CSF promotes the maturation of dendritic cells, in particular in inflammation (20). Neutralizing antibodies against GM-CSF have been successfully used in various models to treat autoimmune or inflammatory diseases, including collagen-induced arthritis (17, 21).

In the present study, the roles of IL-17A and GM-CSF in osteoclast differentiation and activity were investigated *in vitro*, with a focus on the osteoclastogenic potential of GM-CSF-treated OPC. The data demonstrate that IL-17A, in synergism with TNFα and $1,25(OH)_2D_3$, stimulates osteoblasts to release GM-CSF. Exposure of OPC to GM-CSF leads to a dendritic cell-like phenotype. Upon stimulation with M-CSF/RANKL, these cells differentiate into active osteoclasts that are able to dissolve a calcium phosphate (CaP) matrix.

Materials and Methods

Animals. Wild-type (*wt*) *C57Bl/6J* mice and *p55TNFR*$^{-/-}$*/p75TNFR*$^{-/-}$ double knockout mice on *C57Bl/6J* background (22) were bred at the Central Animal Facility of the University of Bern, in compliance with the Swiss guidelines for the care and use of experimental animals. The use of animals in this study was approved by the State Committee for the Control of Animal Experimentation (Bern, CH; permit numbers BE23/13 and BE54/16 to WH).

Cell preparations and cultures. Primary murine osteoblasts were isolated by sequential collagenase digestion of calvariae from 1–2-day-old mice. Briefly, 25 calvariae were digested for 5 × 20 min with 3 mg/ml collagenase type II (Worthington, NJ, USA) in Hank's balanced salt solution (Sigma-Aldrich, Buchs, CH). Fractions containing osteoblasts were pooled and seeded into 75-cm^2 culture flasks (10^6 cells/flask) for culture in medium (αMEM [Gibco/LuBio Science, Lucerne, CH] supplemented with 10% fetal bovine serum [FBS; Sigma-Aldrich] and penicillin/streptomycin at 100 U/ml and 100 µg/ml, respectively [Gibco]). After 4 days, the cells were aliquoted (10^6 cells/ml) and stored in liquid nitrogen. Before the experiments, the cells were expanded in culture medium for 4 days.

BMC were obtained from femora and tibiae of 6- to 8-week-old *C57Bl/6J* mice. The cells were centrifuged at 250 ×g at 4°C for 7 min, and the pellet was resuspended in cell culture medium supplemented with M-CSF (30 ng/ml; Chiron, Emeryville, USA) and incubated (37°C, humidified atmosphere, 5% CO_2) in 75-cm^2 cell culture flasks (4 × 10^6 cells/flask). After 24 h, the non-adherent cells (3 × 10^4 cells/well) were grown with primary osteoblasts (2 × 10^3 cells/well) in 48-well plates. The culture medium was

supplemented with 1,25$(OH)_2D_3$ (10^{-8} M; Sigma-Aldrich), recombinant murine TNFα (0.5 ng/ml; Genentech, San Francisco, USA), and IL-17A (0.1, 1, 5, 10, or 50 ng/ml; PeproTech, London, UK). After 3 days, the medium was replaced, and multinucleated osteoclasts were visible after 4 to 5 days.

Inhibitory antibodies (BD, Allschwil, CH) were used to assess the effects of TNFα and GM-CSF on osteoclastogenesis. Rat anti-mouse antibodies against TNFα and GM-CSF were added to the cultures at 1 µg/ml. Control cultures were treated with the respective isotype controls (rat IgG1 κ Isotype Ctrl and rat IgG2a κ Isotype Ctrl). In cultures with anti-TNFα, RANKL (10 ng/ml, recombinant human RANKL, PeproTech) was added to allow osteoclast formation.

Osteoclast development *in vitro.* Non-adherent, M-CSF-dependent OPC were seeded into 5-cm petri dishes (2×10^6 cells/dish) and grown in αMEM/10% FBS supplemented with M-CSF (30 ng/ml), GM-CSF (0.1, 1, or 10 ng/ml; BD), and M-CSF/ GM-CSF. After 3 days, the cells were detached by incubating them in 0.02% EDTA (Applichem, Darmstadt, Germany) in PBS (Dulbecco's phosphate-buffered saline, pH 7.4; Sigma-Aldrich) at 37°C for 10 min. The cells were seeded into new culture dishes at a density of 10^5 cells/ml. The medium was supplemented with M-CSF (30 ng/ml) and RANKL (20 ng/ml).

GM-CSF ELISA. GM-CSF release was quantitated in the supernatants of cultures treated with IL-17A, TNFα, and 1,25$(OH)_2D_3$ using a BD OptEIA^{TM} Mouse GM-CSF ELISA set (BD) per the manufacturer's recommendations. The absorption was

measured at 450 nm (690 nm reference wavelength) on an Infinite 200Pro Spectrophotometer (Tecan Group Ltd., Männedorf, CH).

Staining of osteoclast cultures for tartrate-resistant acid phosphatase (TRAP). To visualize osteoclasts, cultures were stained for the enzyme TRAP using a commercial kit (Sigma-Aldrich). Before TRAP staining, cultures were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany). TRAP-positive, multinucleated cells (≥ 3 nuclei) were counted as osteoclasts. TRAP activity was quantified in cell lysates. Cells were lysed in 0.1% Triton X-100/1 M NaCl and frozen at -20°C. Fifty microliters of para-nitro-phenyl-phosphate (Sigma-Aldrich; 4.61 mg/ml) was added to 50 µl of cell lysate in 40 mM Na-tartrate/50 mM Na-acetate, pH 4.8, and incubated at room temperature. After 25 min, the reaction was stopped with 50 µl 0.2 M NaOH and absorption was measured at 405 nm (690 nm reference wavelength), using an Infinite 200Pro Spectrophotometer.

RNA isolation and quantitative reverse-transcription (qRT-)PCR. Quantitative PCR was used to assess gene expression in cultures treated with IL-17A, TNFα, and $1,25(OH)_2D_3$. Total RNA was isolated on day 5 of culture, using a NucleoSpin® RNA Kit (Macherey-Nagel, Oensingen, CH) per the manufacturer's protocol. Total RNA was reverse-transcribed with M-MLV reverse transcriptase (Promega, Dübendorf, CH), using random primers (Promega), nucleotide mix (Roche, Basel, CH), and RNase inhibitors (Roche). PCR was carried out on an ABI7500 System (Applied Biosystems), using Assays-on-Demand (AoD; Applied Biosystems/LuBio Science) and TaqMan® Fast Universal PCR Master Mix (Applied Biosystems). After a denaturing period of 20 s at 95°C, 45 amplification cycles of 3 s at 95°C and 30 s at 60°C were performed. The following AoD were used: osteoclast stimulatory transmembrane protein (OC-STAMP;

Mm00512445_m1), dentrocyte expressed seven transmembrane protein (DC-STAMP; Mm04209236_m1), integrin alpha X (CD11c; Mm00498698_m1), colony-stimulating factor 1 receptor (CSF-1R/cFMS; Mm01266652_m1), colony-stimulating factor 2 receptor, alpha, low-affinity (Csf2rα/GM-CSF-Ra; Mm00438331_g1), nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (Nfatc1; Mm00479445_m1), calcitonin receptor (CTR; Mm00432271_m1), adhesion G protein-coupled receptor E1 (F4/80; Mm00802529_m1), and for normalization, β-glucuronidase (GUSB; Mm01197698_m1).

Osteoclast activity assay. The activity of osteoclasts was assessed by their capacity to dissolve amorphous CaP. As described previously (23), sterile-filtered solutions of 0.12 M Na_2HPO_4 and 0.2 M $CaCl_2$ in 50 mM Tris/HCl, pH 7.4, were left in an atmosphere of 5% CO_2 at 37°C overnight. Then, $^{45}[Ca]Cl_2$ (PerkinElmer, Schwerzenbach, CH) was added to a final concentration of 8000 Bq per well. Equal volumes were mixed in 50 ml tubes and CaP precipitated. The slurry was washed with water twice, resuspended with 10 ml of water per 1 ml of slurry, and 200 µl of the suspension was added into the wells of 48-well plates. The plates were dried at room temperature for 3 days and baked at 80°C for 3 h. Before use, the CaP layers were coated with αMEM/30% FBS overnight. Osteoclasts were generated *in vitro* from OPC as described above. Mature osteoclasts were resuspended in 500 µl of medium per dish and seeded into the CaP-coated wells (50 µl of cell suspension per well) in medium supplemented with M-CSF (30 ng/ml), RANKL (20 ng/ml), and HCl (15 mM). After 24 h, the supernatants were collected and $^{45}[Ca]$ was measured. The cells were lysed for measurement of total TRAP activity.

Magnet-activated cell sorting (MACS) and flow cytometry. GM-CSF-dependent progenitor cells (4×10^8) were incubated with anti-CD11c magnetic beads at 4°C for 15 min and loaded onto columns that were placed in a magnetic cell separator (all from Miltenyi Biotech, Bergisch Gladbach, Germany). Positive (adherent) and negative (non-adherent) cell fractions were collected and counted. The individual cell fractions were differentiated into osteoclasts in culture media supplemented with M-CSF/RANKL as described above. Unsorted cells with and without beads served as controls. To assess the efficiency of the sorting procedure, the cells were analyzed by flow cytometry. After washing of the cells with staining buffer (PBS, 0.02% sodium azide, 2% FCS), they were labeled with FITC-conjugated anti-CD11c antibody (BioLegend, San Diego, CA, USA) for 20 min on ice and analyzed in an LSR II SORP (BD). Isotype-matched antibodies (BD) were used as negative controls and BD™ CompBeads were used for compensation. Data were analyzed using the FLOWJO software, version 10.

EdU proliferation assay. To assess proliferation during osteoclast formation from OPC, cells were labeled with the thymidine analogue EdU (5-ethynyl-2´-deoxyuridine). The Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Invitrogen/LuBio Science) was used, following the manufacturer's protocol. OPC were cultured on cover slips, and on days 3 and 4 of culture, 2 µM EdU was added for 4 h. Then, the medium was replaced with fresh culture medium supplemented with M-CSF/RANKL. Two days later, the cells were fixed and the EdU incorporated into the DNA was fluorescently labeled according to the Click-iT® protocol. Cell nuclei were stained with 5 µg/ml Hoechst 33342 (Sigma-Aldrich) in PBS for 30 min and osteoclasts were visualized by TRAP staining. The percentage of

nuclei with incorporated EdU was assessed by counting EdU-positive nuclei and total nuclei within osteoclasts on microphotographs of an area of 8.4 mm^2 per cover slip.

Statistical analysis. Differences in osteoclast numbers and percentages of proliferating nuclei were evaluated by nonparametric *t*-tests, using IBM SPSS Statistics version 22 for Windows. *P*-values < 0.05 were considered significant.

Results

IL-17A in synergism with TNFα inhibits the formation of osteoclasts in cocultures. To assess the effects of IL-17A, TNFα, 1,25$(OH)_2D_3$, and various combinations thereof on osteoclast formation *in vitro*, *wt* OPC were cocultured with osteoblasts from either *wt* mice or *p55TNFR$^{-/-}$/p75TNFR$^{-/-}$* mice. In cocultures with *C57Bl/6J wt* osteoblasts supplemented with 1,25$(OH)_2D_3$/TNFα, IL-17A inhibited osteoclastogenesis, reducing the number of osteoclasts by ≥90% at 1 ng/ml. In cocultures with *C57Bl/6J wt* osteoblasts in the absence of TNFα, only few osteoclasts developed, and IL-17A enhanced osteoclast formation by a factor of 6, reaching approx. 20% as compared to 1,25$(OH)_2D_3$/TNFα (Suppl. Fig. 1A). In cocultures with osteoblasts from *p55TNFR$^{-/-}$/p75TNFR$^{-/-}$* mice, the inhibitory effect of IL-17A on osteoclast development was largely abolished (Suppl. Fig. 1B).

Concomitant action of TNFα and GM-CSF is required for IL-17A-mediated inhibition of osteoclastogenesis. To assess the specific roles of TNFα and GM-CSF in the IL-17A-mediated inhibition of osteoclastogenesis *in vitro*, blocking antibodies against TNFα and GM-CSF were added to the cocultures. Since only low numbers of osteoclasts were formed in cocultures with osteoblasts from *C57Bl/6J* mice in the absence of TNFα, exogenous RANKL (10 ng/ml) was added together with anti-TNFα antibodies and with the respective controls (Fig. 1A,B).

Blocking of TNFα, in the presence of RANKL, abrogated the inhibition of osteoclastogenesis by IL-17A (Fig. 1A), while in cultures with IgG control antibodies, osteoclast development was inhibited by 95% by IL-17A at 1 ng/ml, irrespective of the presence of RANKL (Fig. 1B,D). In cocultures with media supplemented with TNFα,

without $1,25(OH)_2D_3$, osteoclast formation was reduced to approx. one third, but the inhibitory effect of IL-17A was unabated (Fig. 1B). On the other hand, IL-17A was ineffective in cocultures with media supplemented with $1,25(OH)_2D_3$ alone (Fig 1A,B). Attenuation of the IL-17 effect was also observed upon addition of anti-GM-CSF antibodies to cocultures grown with TNFα and $1,25(OH)_2D_3$ (Fig. 1C). In culture media without exogenous TNFα, IL-17A induced an increase in the number of osteoclasts (Fig. 1C,D).

Osteoblasts from *C57BL/6J* mice release GM-CSF upon treatment with IL-17A, $1,25(OH)_2D_3$, and TNFα. The release of GM-CSF by primary osteoblasts in response to an exposure to IL-17A was assessed in cultures grown with $1,25(OH)_2D_3$ (10^{-8} M)/TNFα (0.5 ng/ml). GM-CSF accumulated in the culture supernatant over 72 h in a time- and dose-dependent manner, and the release was fully blocked by anti-TNFα antibodies (Suppl. Fig. 2).

Characterization of the phenotype of monocyte/macrophage lineage cells exposed to M-CSF, GM-CSF, or M-CSF/GM-CSF. OPC were cultured with M-CSF (30 ng/ml), GM-CSF (0.1, 1, or 10 ng/ml), and various concentrations of M-CSF/GM-CSF, and phenotypic markers were assessed by qRT-PCR. Supplementation of the culture medium with GM-CSF caused a decrease in the level of mRNA encoding the pan-macrophage marker F4/80 (Fig. 2A). Transcript levels of the dendritic cell marker CD11c were dose-dependently increased by exogenous GM-CSF, independently of the presence of M-CSF (Fig. 2B). Similar expression patterns were observed for DC-STAMP (Fig. 2C) and OC-STAMP (Fig. 2D). Levels of cFMS mRNA were dose-dependently decreased with increasing concentrations of GM-CSF (Fig. 2E). CSF2R transcripts were

upregulated by GM-CSF, while the presence of M-CSF prevented this increase (Fig. 2F).

OPC precultured with M-CSF, GM-CSF, or M-CSF/GM-CSF give rise to osteoclasts. OPC were cultured with M-CSF (30 ng/ml), GM-CSF (10 ng/ml), or M-CSF/GM-CSF. The potential of the OPC to develop into osteoclasts was assessed by subsequent culture with M-CSF (30 ng/ml)/RANKL (20 ng/ml). Starting on day 0 of osteoclast culture, cells were collected daily and total RNA was prepared for qRT-PCR.

In M-CSF-treated OPC, F4/80 transcript levels were twofold higher than those in OPC treated with GM-CSF and M-CSF/GM-CSF at day 0. Thereafter, F4/80 transcript levels decreased before an induction at day 5 of the osteoclast culture (Fig. 3A). OPC pretreated with GM-CSF and M-CSF/GM-CSF were characterized by increased mRNA levels of CD11c (Fig. 3B), DC-STAMP (Fig. 3C), and OC-STAMP (not shown). The respective transcript levels decreased over time upon exposure to M-CSF/RANKL. With the development of osteoclasts, the levels of DC-STAMP and OC-STAMP transcripts rebounded, which was also observed in cultures of OPC pretreated with M-CSF. Expression of transcripts encoding the osteoclast transcription factor NFATc1 (Fig. 3D) preceded the expression of OC markers and the development of OC. CTR mRNA levels (Fig. 3E) increased with cell differentiation and correlated with the numbers of osteoclasts (Fig 3F).

Osteoclasts generated from M-CSF, GM-CSF, and M-CSF/GM-CSF-treated OPC dissolve CaP *in vitro.* The capacity of osteoclasts generated from M-CSF, GM-CSF, and M-CSF/GM-CSF dependent OPC to dissolve CaP mineral was assessed by

seeding mature cells onto a layer of ^{45}Ca-spiked amorphous CaP (Fig. 4A). Osteoclasts developed faster from M-CSF- than from GM-CSF- and M-CSF/GM-CSF-treated OPC (Fig. 4B). Upon transfer of mature OPC onto the CaP layers, TRAP activity was the same in all 3 groups in the cell suspension and after 1 h of adherence (Fig. 4C). Within 24 h, total TRAP activity from M-CSF-dependent OPC decreased by 20%, while increasing by more than 50% in cultures of osteoclasts from GM-CSF- and M-CSF/GM-CSF-dependent OPC (Fig. 4C). Cells in the latter cultures released twofold higher amounts of ^{45}Ca than M-CSF-dependent OPC (Fig. 4D). Despite the increased dissolution of CaP, the release of ^{45}Ca per TRAP after 24 h was similar among all osteoclast populations (Fig. 4D).

CD11c^{+}, GM-CSF-dependent OPC give rise to active osteoclasts. To check whether the CD11c^{+} dendritic-like cells or a CD11c^{-} subpopulation of the GM-CSF-dependent OPC give rise to osteoclasts, CD11c^{+} cells were isolated by MACS (CD11c^{bright}). Unsorted cells, CD11c^{dim}, and CD11c^{bright} cells were differentiated into osteoclasts and their capacity to dissolve CaP was assessed. In total GM-CSF-dependent OPC (F1) and in control cells with anti-CD11c magnetic beads (F2), ca. 70% of the cells were CD11c^{+}, with median fluorescence intensities (MFI) of the positive cells at 10,796 and 10,110 respectively. The negatively (flow through) sorted cells were 55% positive (CD11c^{dim}; F3; MFI 6,267), and 98% of the positively (adherent) sorted cells (CD11c^{bright}; F4; MFI 24,978) expressed CD11c (Fig. 5A). All populations gave rise to TRAP-positive, multi-nucleated osteoclasts (Fig. 5B), with similar trends being observed for total TRAP activity (Fig. 5C) and dissolution of CaP mineral (Fig. 5D).

Osteoclasts differentiated from GM-CSF-treated progenitors have a higher proliferation potential than those developed from M-CSF-treated progenitors. To investigate whether the difference in osteoclast development from M-CSF- and GM-CSF-dependent OPC is correlated with cell proliferation, the cells were labeled at day 3 or day 4 with EdU, and nuclear labeling was quantitated at days 5 and 6 of culture. Nuclei were visualized with Hoechst (Fig. 6A,C,E), and mitotic nuclei with EdU (Fig. 6B,D,F). The percentage of labeled nuclei was threefold higher in osteoclasts from GM-CSF- and M-CSF/GM-CSF-dependent OPC than in those from M-CSF-dependent OPC after the addition of EdU to the cultures at day 3, and twofold higher when EdU was added at day 4 (Fig. 6G). After six days in culture, the total number of nuclei was doubled in osteoclasts differentiated from OPC dependent on GM-CSF and M-CSF/GM-CSF as compared to osteoclasts derived from M-CSF-dependent OPC (Fig. 6H).

Discussion

The present study shows that IL-17A in synergy with TNFα and $1,25(OH)_2D_3$ inhibits the formation of osteoclasts *in vitro* through osteoblast-derived GM-CSF. The growth factor directs OPC differentiation towards a dendritic cell-like phenotype. Upon exposure to M-CSF/RANKL, differentiation towards osteoclasts was delayed in GM-CSF-pretreated OPC, allowing proliferation over a longer period, resulting in the expansion of the OPC pools and the development of higher numbers of osteoclasts *in vitro*.

The effects of IL-17A on osteoclastogenesis *in vitro* are indirect, targeting osteoblast lineage cells in cocultures. The cytokine induces an increase in RANKL and a decrease in OPG expression, thus supporting an osteoclastogenic microenvironment (data not shown). Simultaneously, IL-17A in synergy with TNFα and $1,25(OH)_2D_3$ stimulates the release of GM-CSF, which is considered to act as an inflammatory cytokine (17, 24) and as an inhibitor of osteoclast development (14, 15, 25). This specific effect of IL-17A on osteoblast lineage cells contrasts the previously described dual effects of TNFα; inhibiting osteoclast development indirectly by inducing osteoblast lineage cells in synergy with $1,25(OH)_2D_3$ to release GM-CSF (14), while stimulating osteoclastogenesis through a direct effect on OPC (3, 4, 26, 27)**.** Haematopoietic growth factors act in sequence on haematopoietic stem cells and, depending on the factor, cells differentiate towards specific lineages (28, 29). GM-CSF acts on early progenitor cells and blocks their differentiation towards the monocyte/macrophage and osteoclast lineages (15). In the presence of GM-CSF, the cells fail to raise levels of RANK, consequently leading to interruption of RANK/RANKL signaling. Therefore, the addition of exogenous RANKL to cocultures could not abrogate the inhibitory effect of GM-CSF. Several studies have

shown that the inhibition of osteoclastogenesis by GM-CSF prevents the induction of *c-Fos* and *Fra-1*, and indeed, overexpression of c-FOS by retroviral infection rescued osteoclast differentiation despite the presence of GM-CSF (14, 30). In a later differentiation stage, characterized by high levels of RANK, GM-CSF no longer affects osteoclastogenesis (15, 25).

The second part of the study provides evidence that GM-CSF-dependent OPC express a dendritic cell-like phenotype, characterized by the expression of CD11c and DC-STAMP, but not to undergo irreversible commitment to the dendritic cell lineage. Upon exposure to M-CSF/RANKL, CD11c and DC-STAMP transcript levels were decreased in GM-CSF-pretreated OPC, before they developed into osteoclasts and reassumed the expression of DC-STAMP.

The effect of GM-CSF on OPC depends on its concentration. In cultures in the presence of GM-CSF in the picogram range, cells remained $CD11c^-$, even though differentiation to osteoclasts was blocked (14). Treatment of OPC with M-CSF and GM-CSF in the nanogram range increased CD11c expression dose-dependently, with a concomitant loss of the pan-macrophage marker F4/80. The concentration-dependent action of GM-CSF on haematopoietic cells may be caused by differential activation of signaling pathways, analogous to the action of transforming growth factor β (TGFβ) on haematopoietic cells (31).

DC-STAMP is required for the regulation of dendritic cell activity and the maintenance of immune self-tolerance (32, 33), but it is also present on late OPC and, together with OC-STAMP, participates in cell fusion (34, 35). In OPC, levels of DC-STAMP and OC-

STAMP transcripts increased in parallel with those of CD11c. In cultures of GM-CSF-pretreated OPC, DC-STAMP and OC-STAMP mRNA levels decreased and then increased again with osteoclast maturation. This loss of DC-STAMP and OC-STAMP expression during osteoclast culture indicates that GM-CSF pretreated progenitors first lose their dendritic cell-like phenotype before fusing into multinucleated osteoclasts. Based on the expression of osteoclast transcription factor NFATc1 and on the time course of the detection of osteoclasts in culture, osteoclasts from GM-CSF-pretreated OPC do not differ from osteoclasts generated from M-CSF-pretreated OPC, while their differentiation is delayed. This contrasts a recent report postulating that dendritic cells directly fuse to form osteoclasts, so-called DC-OC (36). However, no specific markers for DC-OC, which would allow distinguishing them from “normal” osteoclasts, are available at present. It has been shown that immature dendritic cells generated from human blood monocytes differentiate into functional osteoclasts in the presence of M-CSF and RANKL (37). Furthermore, conventional dendritic cells were found to resolve the osteopetrotic phenotype within one month when injected into osteopetrotic *oc/oc* mice defective in osteoclast resorption (38). The differentiation mechanisms, however, were not elucidated in these studies, and taking into account the time frame before effects were observed, it is likely that these cells underwent dedifferentiation before developing into osteoclasts.

Osteoclasts from GM-CSF-treated OPC showed the same capacity to dissolve mineral as osteoclasts from M-CSF-treated OPC, but are delayed in differentiation. After transfer onto CaP at day 5, TRAP activity further increased, which was in contrast to osteoclasts from M-CSF-pretreated OPC, whose number and TRAP activity decreased

simultaneously. Thus, osteoclasts from GM-CSF-pretreated progenitors had more time to proliferate in the coculture and to leave the cell cycle at a later time point than did M-CSF-pretreated OPC, resulting in a higher number of late progenitors capable of differentiation and fusion. These findings are in accordance with studies demonstrating the proliferative effects of GM-CSF on haematopoietic progenitor cells (39-41).

The present study provides evidence for the action of GM-CSF as a positive modulator of osteoclastic activity in the context of inflammatory disease, despite its direct inhibitory effect *in vitro* on osteoclastogenesis. Furthermore, it confirmed that cells expressing the dendritic cell marker CD11c are not irreversibly committed to the dendritic cell lineage, but can give rise to osteoclasts upon stimulation with M-CSF/RANKL (25, 37, 38). Osteoclasts and dendritic cells derive from the same myeloid progenitors, and differentiation has been shown to be regulated by M-CSF and GM-CSF (30). However, as described above, the action of GM-CSF depends on the timing and the differentiation state of the cells, and GM-CSF overrides M-CSF signaling in early haematopoietic progenitors (39). Besides its systemic effects, GM-CSF secreted by activated T-cells in inflamed joints may also locally increase the number of dendritic cells, which may serve as precursors of osteoclasts and contribute to the exacerbated osteoclastogenesis in RA (42-44).

It has been postulated previously that OPC and osteoclasts may develop and mature in different physical compartments in the organism (14). In circulation, elevated levels of GM-CSF, as they occur in inflammatory diseases, act by increasing the proliferation of haematopoietic progenitor cells (45). It was reported that the pool of $CD11c^+$ cells is expanded in RA patients (46, 47). Upon recruitment to the bone, the composition of the

haematopoietic microenvironment changes; RANKL from activated T-cells and osteoblasts takes over and locally induces the development of osteoclasts (7).

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Figure Legends

Figure 1. *Effects of IL-17A, TNFα, and 1,25(OH)$_2$D$_3$ on the development of osteoclasts in cocultures of wt osteoblasts and OPC*. Cells were cultured with or without IL-17A, TNFα, and 1,25(OH)$_2$D$_3$ and stained for TRAP after 5 days. Addition of IL-17A to cocultures with TNFα and 1,25(OH)$_2$D$_3$ resulted in a dose-dependent inhibition of osteoclast formation. Neutralizing antibodies against TNFα and GM-CSF attenuated the inhibitory effect of IL-17A. A**,** anti-TNFα/RANKL; B, IgG1 κ Isotype control/RANKL; C, anti-GM-CSF; D, IgG2a κ Isotype control. Bars show the mean ± SD (n = 6).

Figure 2. *Effects of M-CSF, GM-CSF, and M-CSF/GM-CSF on gene expression by OPC*. Levels of transcripts coding for F4/80 (A), CD11c (B), DC-STAMP (C), OC-STAMP (D), cFMS (E), and CSF2R (F) were quantitated by qRT-PCR and normalized to levels of transcripts encoding GUSB. GM-CSF dose-dependently upregulated CD11c, a marker for dendritic cells, and downregulated the pan-macrophage marker F4/80. DC-STAMP and OC-STAMP mRNA levels were elevated with increasing doses of GM-CSF. Bars show the mean ± SD (n = 3).

Figure 3. *OPC, pretreated with M-CSF, GM-CSF, or M-CSF/GM-CSF, give rise to osteoclasts.* OPC were cultured with M-CSF (30 ng/ml; white bars), GM-CSF (10 ng/ml; grey bars), or M-CSF/GM-CSF (black bars) for 3 days. The osteoclastogenic potential of the three OPC populations was assessed by culturing the cells in media supplemented with M-CSF/RANKL. Levels of transcripts encoding F4/80 (A), CD11c (B), DC-STAMP (C), NFATc1 (D), and CTR (E) were quantitated and osteoclasts were counted (F). GM-CSF, alone or in combination with M-CSF, caused an increase in CD11c and DC-STAMP transcripts. The levels of CD11c and DC-STAMP mRNA decreased gradually

after starting M-CSF/RANKL treatment, while DC-STAMP mRNA levels increased again in parallel to osteoclast development, as was observed in M-CSF treated OPC. Osteoclast formation was delayed in cultures of GM-CSF- and M-CSF/GM-CSF-treated OPC. (A) – (E)**:** bars show the mean ± SD (n=3); (F): bars show the mean ± SD (n = 6).

Figure 4. *Osteoclasts generated from OPC pretreated with M-CSF, GM-CSF, and M-CSF/GM-CSF dissolve amorphous CaP.* A, Osteoclasts on the mineral were stained for TRAP followed by phase-contrast imaging. The resorbed surfaces are visible as bright areas. Scale bar = 100 µm. B, Osteoclastogenesis from the OPC populations was followed over time. C, Total TRAP activity was measured after 0 h (before seeding onto CaP), after 1 h (adhesion), and after 24 h. D, Release of ^{45}Ca after 24 h was normalized to total TRAP activity. Bars show the mean ± SD (n = 6).

Figure 5. *CD11c-positive cells give rise to osteoclasts.* OPC were treated for 3 days with GM-CSF, and $CD11c^{+}$ cells were sorted by MACS. The potential of the cell fractions (non-sorted, $CD11c^{bright}$, $CD11c^{dim}$) to develop into osteoclasts was assessed by culturing the cells in media supplemented with M-CSF/RANKL. After sorting, the cells were analyzed by flow cytometry. A, The $CD11c^{bright}$ were nearly 100% positive for CD11c. After 5 days, the cells were stained for TRAP. B, All fractions gave rise to osteoclasts. C, Osteoclast development over time was similar for all 4 OPC populations as assessed by total TRAP activity in the cultures (days 3, 4, 5, 6 from white bars to black bars). D, Release of ^{45}Ca, normalized to TRAP activity after 1 h (white bars), 12 h (grey bars), and 24 h (black bars). Osteoclasts from all OPC populations were able to dissolve CaP. F1: OPC, F2: unsorted cells, incubated with magnetic beads, F3: $CD11c^{dim}$, F4: $CD11c^{bright}$. Bars show the mean ± SD (n = 6).

Figure 6. *Proliferation of OPC pretreated with M-CSF, GM-CSF, and M-CSF/GM-CSF during osteoclastogenesis*. OPC were precultured with M-CSF (white bars), GM-CSF (grey bars), and M-CSF/GM-CSF (black bars) and subsequently grown in media supplemented with M-CSF/RANKL. On days 3 and 4, the cultures were labeled with EdU for 4 h. Two days later, the cultures were stained with Hoechst dye and for EdU. Arrows indicate Hoechst-positive nuclei within osteoclasts, positive or negative for EdU. Scale bar = 100 µm. A, M-CSF-pretreated OPC, Hoechst staining and phase contrast; B, M-CSF-pretreated OPC, EdU staining and phase contrast; C, GM-CSF-pretreated OPC, Hoechst staining and phase contrast; D, GM-CSF-pretreated OPC, EdU staining and phase contrast; E, M-CSF/GM-CSF-pretreated OPC, Hoechst staining and phase contrast; F, M-CSF/GM-CSF-pretreated OPC, EdU staining and phase contrast; G, Proportion of EdU-positive to total nuclei; H, Total number of nuclei within osteoclasts. Six pictures per well were evaluated, corresponding to an area of 8.4 mm^2. Bars show the mean ± SD of triplicate cultures. ***P≤ 0.05, **P≤ 0.01, *P≤ 0.001 versus M-CSF-treated OPC culture.

Supplementary figures

Suppl. Figure 1. *TNFα is required for IL-17A mediated inhibiton of osteoclast formation.* A, Effects of IL-17A, TNFα, and 1,25$(OH)_2D_3$ on the development of OC in cocultures of *wt* OB and OPC. The addition of IL-17A to cultures supplemented with TNFα and 1,25$(OH)_2D_3$ resulted in a dose-dependent inhibition of OC formation. In the absence of TNFα, 1,25$(OH)_2D_3$-mediated OC formation was stimulated by IL-17A. In the absence of 1,25$(OH)_2D_3$, no OC did form. B, Effect of IL-17A on the development of OC in cocultures of *p55TNFR*$^{-/-}$*/p75TNFR*$^{-/-}$ OB and *wt* OPC. The inhibitory effect of IL-17A in the presence of TNFα and 1,25$(OH)_2D_3$ was abolished. Cultures were stained for TRAP on day 5 and osteoclasts were counted. Bars show the mean ± SD (n = 6).

Suppl. Figure 2. Osteoblasts release GM-CSF upon stimulation with IL-17A, TNFα, and 1,25$(OH)_2D_3$. GM-CSF protein levels in the culture supernatants were quantified by ELISA. *Wt* OB were cultured with different concentrations of IL-17A in the presence of TNFα and 1,25$(OH)_2D_3$ for 2, 4, 8, 24, and 72 h. IL-17A dose-dependently increases the levels of GM-CSF released into the medium. Addition of anti-TNFα (1 ng/ml) prevented GM-CSF release. Bars show the mean ± SD of triplicate cultures. Ab = anti-TNFα antibodies.

Figure 1

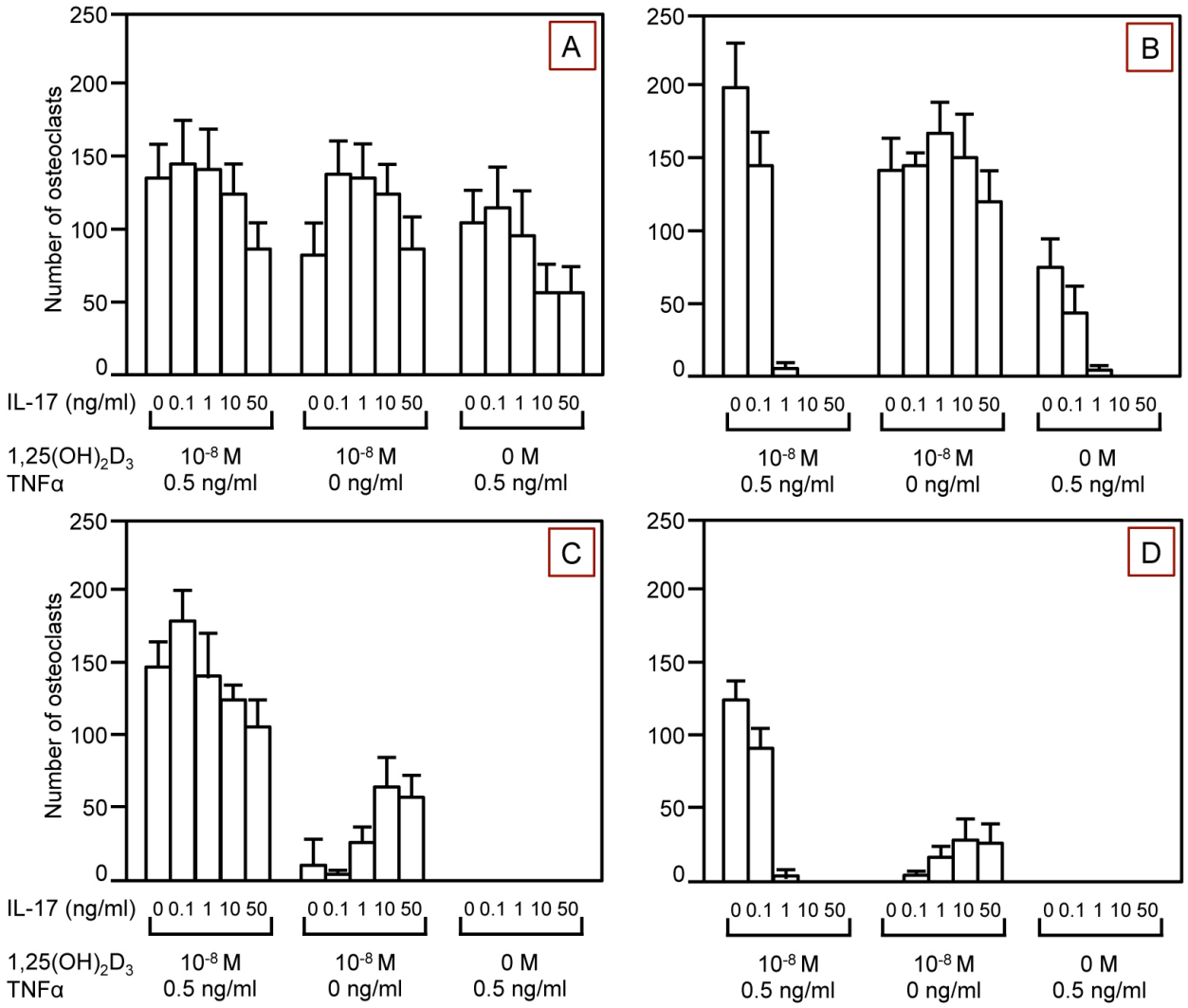

A
B
C
D
Number of osteoclasts
250
200
150
100
50
0
IL-17 (ng/ml)
0 0.1 1 10 50
1,25(OH)2D3
TNFα
10-8 M
0.5 ng/ml
10-8 M
0 ng/ml
0 M
0.5 ng/ml

Figure 2

Add Gene Names to Figures

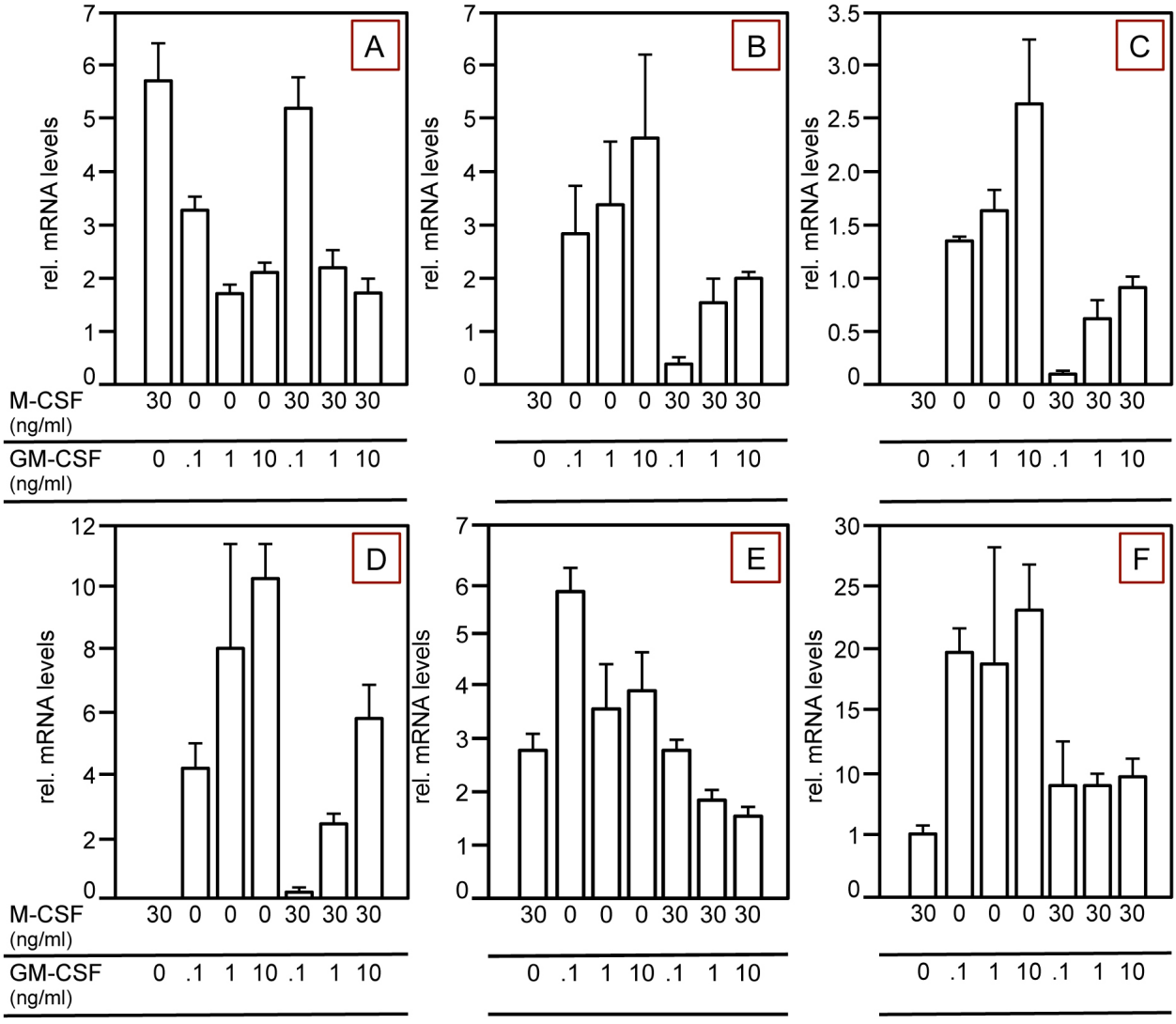


Figure 3

Add Gene Names to Figures

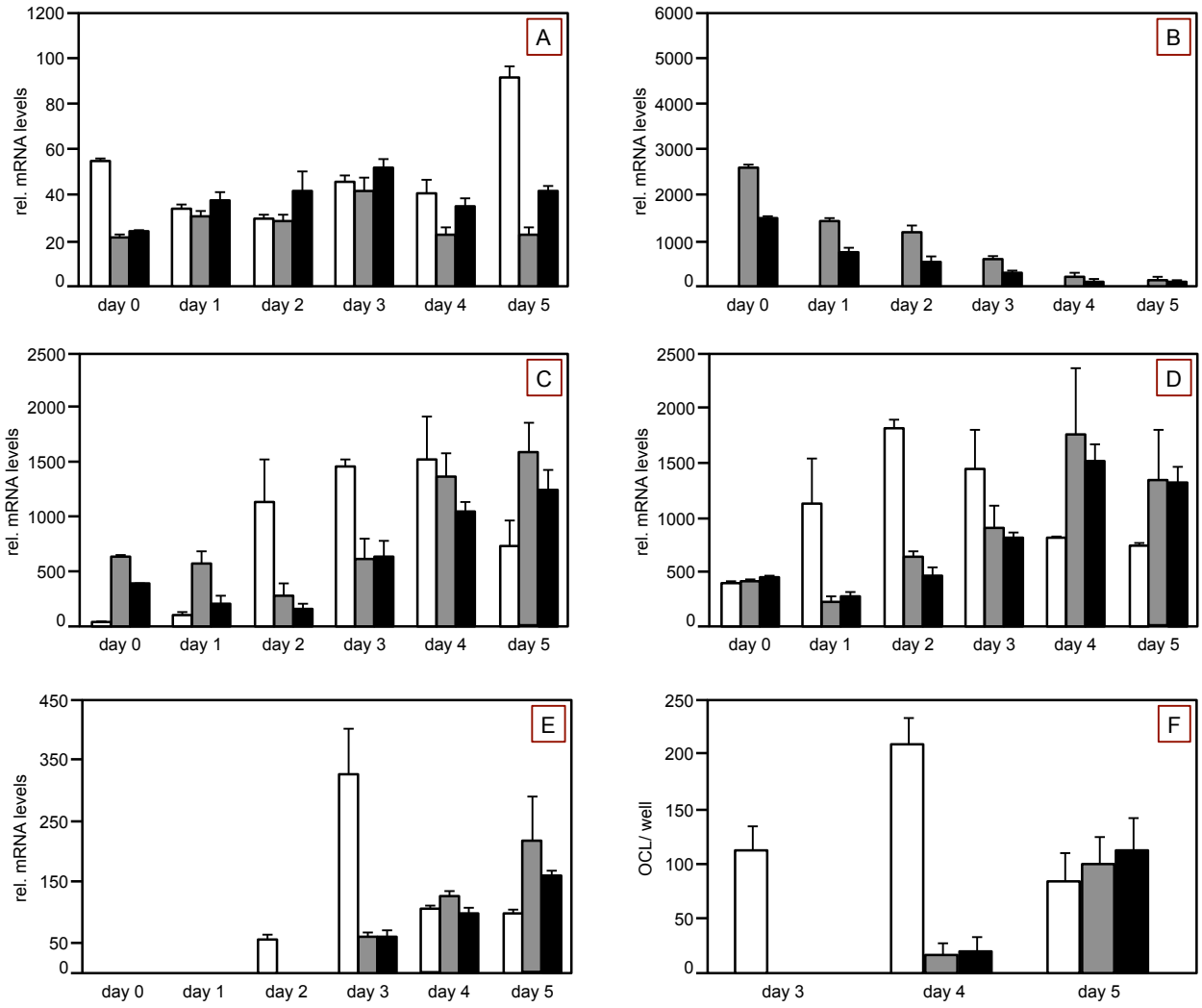


Figure 4

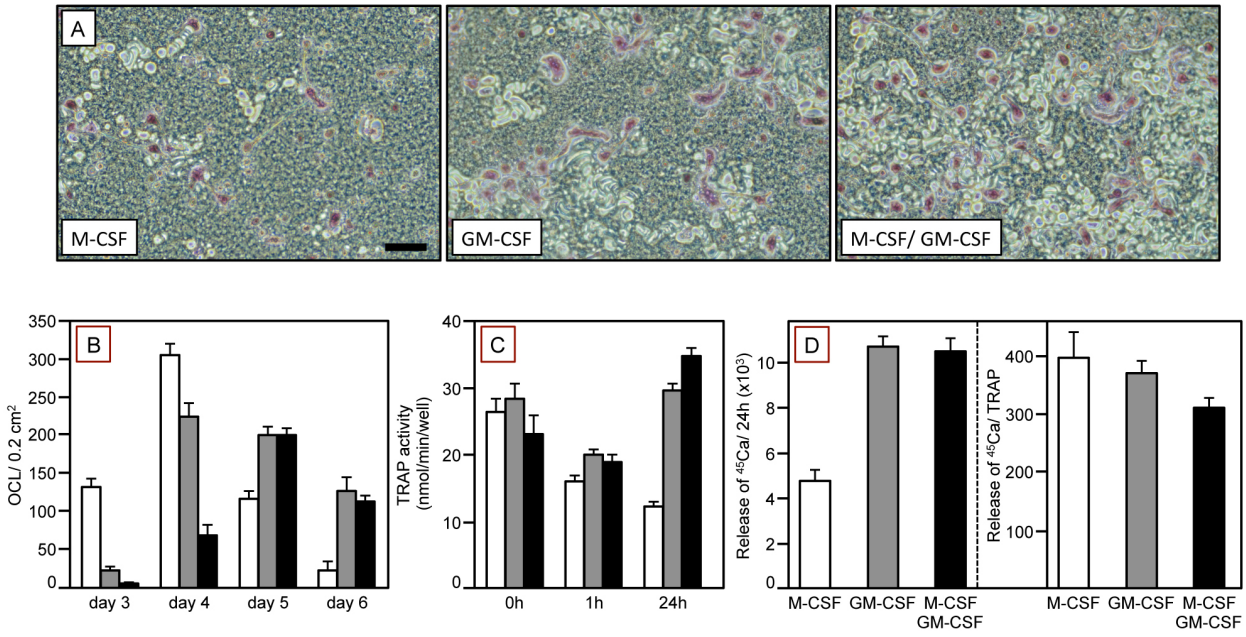

A
M-CSF
GM-CSF
M-CSF/ GM-CSF
B
OCL/ 0.2 cm²
day 3
day 4
day 5
day 6
C
TRAP activity (nmol/min/well)
0h
1h
24h
D
Release of ⁴⁵Ca/ 24h (x10³)
Release of ⁴⁵Ca/ TRAP
M-CSF
GM-CSF
M-CSF GM-CSF

Figure 5

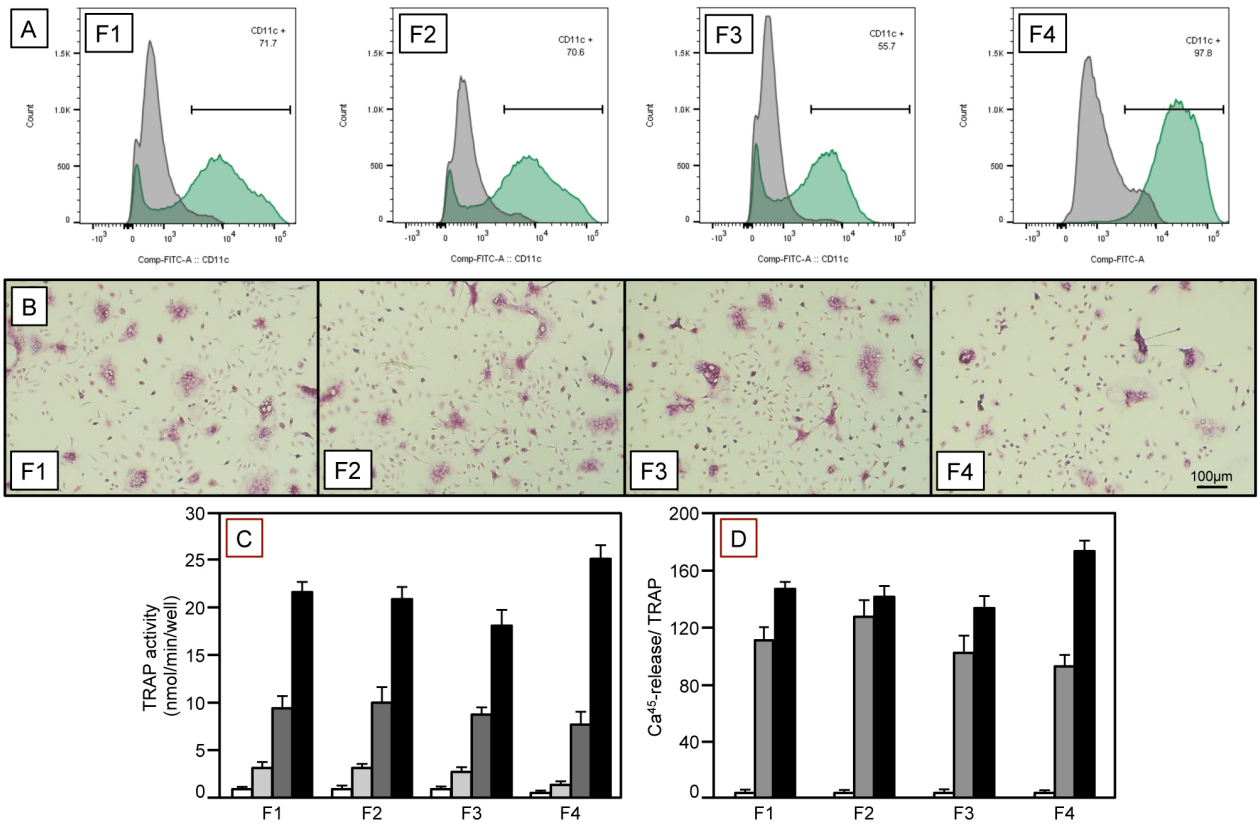
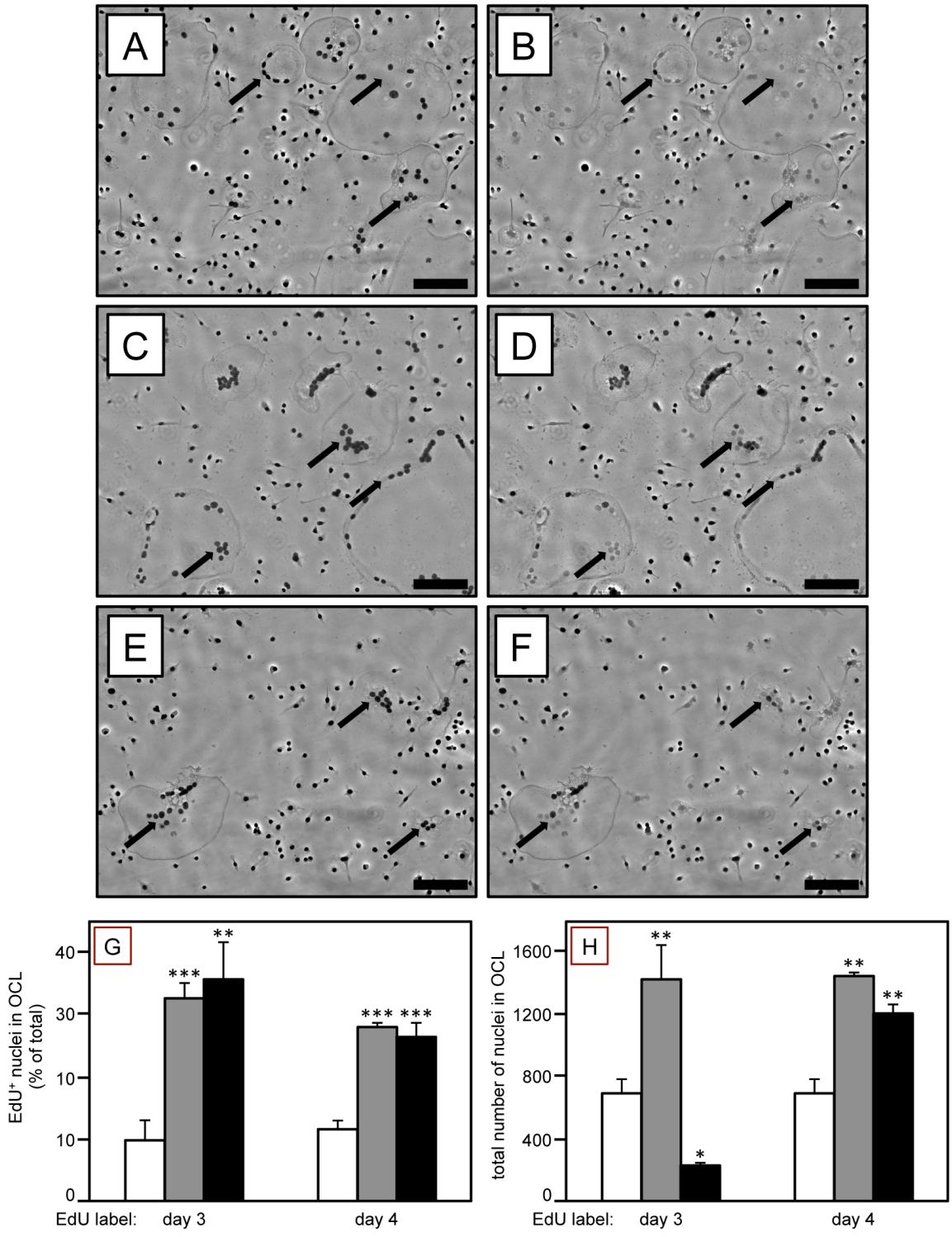
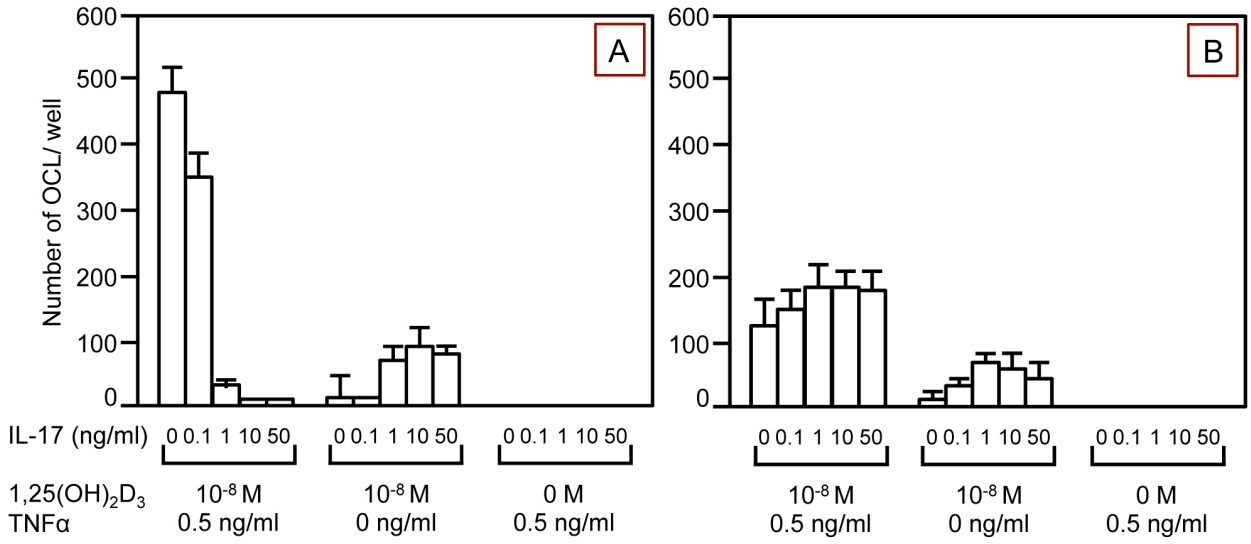

A
F1
CD11c +
71.7
F2
CD11c +
70.6
F3
CD11c +
55.7
F4
CD11c +
97.8
Count
Comp-FITC-A :: CD11c
Comp-FITC-A
B
F1
F2
F3
F4
100µm
C
TRAP activity
(nmol/min/well)
D
Ca45-release/ TRAP

Figure 6


A
B
C
D
E
F
G
EdU⁺ nuclei in OCL (% of total)
40
30
10
10
0
***
**
*** ***
EdU label:
day 3
day 4
H
total number of nuclei in OCL
1600
1200
800
400
0
**
*
**
**
EdU label:
day 3
day 4

Suppl. Figure 1



Suppl. Figure 2